REMARKS

It is respectfully requested that the present Reply be entered into the Official File in view of the fact that the Reply automatically places the application in condition for allowance. Thus, the present Reply is believed to be in proper form for placing the application in condition for allowance.

The amended claim presents no new issues requiring further search or consideration because a claim of the same or similar scope has previously been presented and subsequently examined. The amendment to claim 33 actually deletes subject matter.

In the alternative, if the Examiner continues with the rejections of the present application, it is respectfully requested that the present Reply be entered for purposes of an Appeal. The Reply reduces the issues on appeal by overcoming the rejection under 35 U.S.C. § 112, first paragraph. Thus, the issues on appeal would be reduced.

Status of Claims

Applicants respectfully request the Examiner to reconsider the present application in view of the foregoing amendments to the claims.

In the present Reply, claim 33 has been amended herein. Claims 1-32, 34-36 and 39-60 were previously canceled without prejudice or disclaimer of the subject matter contained therein. Thus, claims 33, 37, 38 and 61-63 are pending in the present application. No new matter has been added by way of the amendment to claim 33, since this amendment has support throughout the present specification (see, e.g., page 2, lines 11+).

Based upon the above considerations, entry of the present amendment is respectfully requested.

In view of the following remarks, Applicants respectfully request that the Examiner withdraw all rejections and allow the currently pending claims.

Issues Under 35 U.S.C. § 112, First Paragraph

Claims 33, 38 and 61-63 stand rejected under 35 U.S.C. § 112, first paragraph for asserted lack of enablement (see pages 2-3 of the Office Action). This rejection is respectfully traversed. Reconsideration and withdrawal thereof are respectfully requested.

The Examiner indicates that only anemia is not enabling in the Office Action. Thus, Applicants respectfully refer the Examiner to the scope of the claims as presented herein. It is believed that this rejection has been overcome or rendered moot. Reconsideration and withdrawal of this rejection are respectfully requested.

Issues Under 35 U.S.C. § 103(a) (pages 3-4 of the Office Action)

Claims 33, 37, 38 and 61-63 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Sakai et al. '004 (WO 96/34004) and Lion Corp. '247 (JP 11-21247), as set forth in the previous Office Action. Applicants respectfully traverse, and reconsideration and withdrawal thereof are respectfully requested.

Overall, the Examiner is maintaining the rejection in view of Sakai '004 and Lion '247 because, in part, the fucoidan of the present invention "has similar activity with the fucoidan derived

from *Fucus vesiculosus*, one of LION's preferred fucoidans" (at page 4, lines 15-17 of the Office Action). These comments, as well as some of the other Examiner's concerns, are addressed below.

Differences in fucoidans

In general, fucoidan is a generic term for a polysaccharide comprising a sulfated fucose as a constituting saccharide, contained in Phaephyceae, Echinodermata, or the like. The fucoidans of different origins are known to have different chemical structures. In other words, those fucoidans of different origins only have in common a polysaccharide comprising sulfated fucose as a constituting saccharide. But a fucoidan with a different origin will have different structure from a fucoidan of another origin. Such differences are especially true for those fucoidans derived from marine algae since those fucoidans have very complicated structures due to a) the existence of the constituting saccharides other than fucose, b) the presence or absence of branched structure, c) the presence of absence of acetylation, d) added position of the sulfated group, etc., these structures have not yet been elucidated at present. To support Applicants' position, enclosed herewith as "Exhibit 1" is a copy of *Carbohydrate Research* ("Structure of a fucoidan from the brown seaweed *Fucus evanescens* C.Ag.," Vol. 339, pp. 719-730 (2002)), showing the contents mentioned above.

Structural differences leading to physiological differences

As a result of such structural differences, not all the fucoidans will necessarily have the same physiological activities. As an example, a fucoidan derived from *Laminaria japonica* versus a fucoidan derived from Sargassum do not share the same therapeutic effects for treating

an allergy (hyaluronic acid synthesis promoting action). Therefore, not all compounds, which are polysaccharides comprising sulfated fucose, exhibit therapeutic effects for an allergy.

Structural and physiological differences in fucoidans relating to cited references

As mentioned, a fucoidan derived from *Laminaria japonica* versus a fucoidan derived from Sargassum do not share the same therapeutic effects for treating an allergy. This is especially true regarding the fucoidan of the cited Lion '247 reference. According to Lion '247, the therapeutic effects are not found in the fucoidan derived from *Laminaria iavonica*, which is a very close species to *Kiellmaniella crassifolia* as used in the present invention. In Lion, at paragraphs [0005]-[0007], this reference describes that fucoidans extracted from Laminariales and Sargassum belonging to Phaeophyceae had weaker effects (of hyaluronic acid synthesis promoting action), which shows that the effects of the present invention are specific to the fucoidan derived from a specified marine alga recited in the claim. Also, Lion '247 discloses that fucoidan derived from *Laminaria japonica* and fucoidan derived from Sargassum do not have hyaluronic acid synthesis promoting action (Test Example 1), that these fucoidans have low hyaluronidase activity inhibitory action (Test Example 2), and that these fucoidans have low histamine release suppressive action (Test Example 3) (which is why Applicants maintain that Lion '247 teaches away from achieving the present invention by disclosing against the effectiveness of the treatment of an allergic disease of the fucoidan derived from *Kjellmaniella crassifolia* (Laminariales)). Thus, there are not only structural differences based on the origin of the particular fucoidan, but physiological differences as well.

Applicants note that this rejection is being maintained from the previous Office Action. In the previous Office Action (of May 6, 2004), the Examiner refers to a fucoidan derived from *K. crassifolia* (page 5 of the Office Action) and fucoidans from brown algae (Phaeophta) (referring to the Sakai references; at page 6 of the Office Action). However, as mentioned above, the fucoidan origin can lead to structural and physiological differences. The present invention is directed to a method of treating an autoimmune disease, diabetes, septic shock, inflammatory enteropathy, chronic articular rheumatism, multiple sclerosis, uveitis or an allergic disease, wherein the method comprises administering a fucoidan derived from *Kjellmaniella crassifolia* and/or a degradation product thereof. Thus, the present invention uses a different fucoidan that what is discussed in Sakai '004.

In view of the above, even if the fucoidan were a polysaccharide containing sulfated fucose, one of ordinary skill in the art cannot easily deduce, and it would not be obvious as to whether or not a fucoidan derived from *Kjellmaniella crassifolia* has the same therapeutic effects for allergy as those described in Sakai '004 and Lion '247. Such a finding has been accomplished for the first time in the present invention.

Applicants also submit that "Obviousness requires one of ordinary skill in the art have a reasonable expectation of success as to the invention — 'obvious to try' and 'absolute predictability' are incorrect standards." *Velander v. Garner*, 68, USPQ2d 1769, 1784 (Fed. Cir. 2003) (citing *In re O'Farrell*, 853 F.2d 894, 903, 7 USPQ2d 1673 (Fed. Cir. 1988)). In this regard, Applicants respectfully submit that the requisite reasonable expectation of success is lacking since a reliance on, e.g., disclosure regarding fucoidans from brown algae (Phaeophta), is an improper invitation to experiment or using an obvious to try rationale. *See In re Vaeck*, 947

F.2d 488, 493, 20 USPQ2d 1438, 1442 (Fed. Cir. 1991). More specifically, relying on the generic disclosure of brown algae (Phaeophta) (as disclosed in Sakai '004) or the fucoidan derived from *Laminaria iavonica* (in Lion '247) to equal the present invention is instead merely an (improper) invitation to experiment so that one of ordinary skill in the art could eventually and maybe achieve the present invention. Thus, the requisite reasonable expectation of success is lacking.

Further, the mere fact that disclosures can be combined does not make the combination obvious unless the art also contains something to suggest the desirability of the combination. *See, In re Gordon*, 221 USPQ 1125, 1127 (Fed. Cir. 1984) and *In re Imperato*, 179 USPQ 730, 732 (CCPA 1973). That suggestion is lacking here in the cited references so that one of ordinary skill in the art would not be properly motivated in achieving the present invention. Instead, the skilled artisan would only be motivated to experiment with, e.g., brown algae and not achieve the present invention.

Applicants also submit that the other requirement of disclosure of all claimed features for a *prima facie* case of obviousness has not been satisfied. Besides the specific diseases or conditions recited, Applicants submit that the cited combination still lacks disclosure of the claimed fucoidan (or degradation product thereof). *See In re Vaeck.*

Thus, Applicants respectfully submit that a *prima facie* case of obviousness has not been established for the reasons stated above. Further, Applicants respectfully submit the present invention is not obvious from the cited combination of Sakai '004 and Lion '247 as asserted in the Office Action. Accordingly, reconsideration and withdrawal of this rejection are respectfully requested.

Issues Under 35 U.S.C. § 103(a) (pages 4-5 of the Office Action)

Claims 33, 38 and 61-63 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Sakai '004 (WO 96/34004) and McCaffrey et al. (*Biochem. Biophys. Res. Comm.*, 1992) in view of Grainger et al. '911 (U.S. 6,117,911). Applicants respectfully traverse, and reconsideration and withdrawal of this rejection are respectfully requested based on the following.

As discussed above, there are not only structural differences based on the origin of the particular fucoidan, but physiological differences as well.

The Examiner cites the secondary reference of McCaffrey as disclosing an activation action of TGF-β by the fucoidan. According to page 775, lines 10-13 of McCaffrey, this activation is an action by the fucoidan derived from *Fucus vesiculasus*, and actions for fucoidans derived from other marine algae have neither been disclosed nor suggested. In the same manner as the arguments for the rejection regarding the combination of Sakai '004 with Lion '247 as discussed above, the fucoidan derived from *Fucus vesiculasus* (of McCaffrey) and the fucoidan derived from *Kjellmaniella crassifalia* are compounds having different chemical structures. Therefore, Applicants respectfully submit that it is not readily obvious to one of ordinary skill in the art that the fucoidan derived from *Kjellmaniella crassifalia* has an activating action of TGF-β due to the different origins of the fucoidans. Thus, Applicants respectfully submit that a *prima facie* case of obviousness has not been established.

Further, the fucoidan derived from *Kjellmaniella crassifalia* and the fucoidan derived from *Fucus vesiculasus* are compounds having different chemical structures according to a recent study made by one of the inventors of the present invention (see Exhibit 2 which is

enclosed - *Baiasaiensu ta Indasutarii (Biasience and Industry),* "Structures and biological activities of marine algal fucoidans and their oligosaccharides," Vol. 60(6) (2002)). (Exhibit 2 is in a foreign language, but a portion is translated as discussed below). This is further proof of Applicants' position.

Specifically, Exhibit 2 discusses the structure of the fucoidan oligosaccharides obtained by enzyme digestion, wherein the fucoidans are derived from marine algae, showing three kinds of fucoidans derived from *Kjellmaniella crassifalia* (sulfated fucoglucuronomannan, sulfated fucogalactan, and sulfated fucane) and two kinds of fucoidans derived from *Fucus vesiculasus* (sulfated fucoglucuronomannan and sulfated fucane) (see Figure 2, p.379, of the attached Exhibit 2). Figure 2 of Exhibit 2 is translated into English hereinbelow as follows.

Sulfated Fucoglucuronomannan Derived from *Kjellmaniella crassifalia*

Main Oligosaccharide	ΔGAl-2(F(3S)α1-3)Manα1-4(GAβ1-2(F(3S)α1-3)Man)n	n=0, 1
Main Structure of Polysaccharide	(-4Gaβ1-2(F(3S)α1-3)Manα1-)m	

Sulfated Fucoglucuronomannan Derived from *Fucus vesiculasus*

Main Oligosaccharide	ΔGA1-2(Ff(5S)α1-4F(2,3diS)α1-3)Man(6S)
Existing Form in Polysaccharide	-4GAl-2(Ff(5S)α1-4F(2,3diS)α1-3)Man(6S)α1-

Sulfated Fucogalactan Derived from *Kjellmaniella crassifolia*

Main Oligosaccharide	Gal(3S)β1-6Gal(3S)β1-6(F(3S)α1-4F(3S)α1-3Galβ1-4)Gal(3S)
Existing Form in Polysaccharide	-6Gal(3S)β1-6Gal(3S)β1-6(F(3S)α1-4F(3S)α1-3Galβ1-4)Gal(3S)β1-

Sulfated Fucane Derived from *Kjellmaniella crassifolia*

Main Oligosaccharide	F(2,4diS)α1-3F(2,4diS)α1-3(F(3S)α1-2)F(4S)α1-3F(2,4diS)α1-3F(2,4diS)α1-3F(2,4diS)

Sulfated Fucane Derived from *Fucus vesiculosus*

Main Oligosaccharide	Fα1-3(F(2S)α1-4F(2,3diS)α1-3)nF(2S)	n=1, 2, 3, 4

Figure 2 Structure of Fucoidan Oligosaccharide Obtained by Enzyme Digestion and Entire Structure of Fucoidan

[Abbreviations in the drawings: F, L-fucose, Ff, L-fucofuranose; GA, D-glucuronic acid; ΔGA, 4,5-unsaturated D-glucuronic acid; Gal, D-galactose; Man, D-mannose; S, o-sulfate]

Applicants note that it is described in Exhibit 2 that the main chain structure of the sulfated fucoglucuronomannan is the same between the fucoidan derived from *Kjellmaniella crassifolia* and the fucoidan derived from *Fucus vesiculosus,* but the side chain structures are different from each other. Also, the main chain of the sulfated fucane derived from *Kjellmaniella crassifolia* is structurally different from that derived from *Fucus vesiculosus.* Therefore, it is evident that the fucoidan derived from *Kjellmaniella crassifolia* and the fucoidan derived from *Fucus vesiculosus* are structurally different compounds from each other. Thus, the cited

combination of references is improper since one of ordinary skill in the art would not be motivated and/or reasonably expect to be successful in achieving the present invention since different fucoidans are used in the cited references.

Thus, Applicants respectfully submit that a *prima facie* case of obviousness has not been established for the reasons stated above. Further, Applicants respectfully submit the present invention is not obvious from Sakai '004, McCaffrey and Grainger '911 as asserted in the Office Action. Accordingly, reconsideration and withdrawal of this rejection are respectfully requested.

CONCLUSION

A full and complete response has been made to all issues as cited in the Office Action. Applicants have taken substantial steps in efforts to advance prosecution of the present application. Thus, Applicants respectfully request that a timely Notice of Allowance issue for the present case.

If the Examiner believes that personal communication will expedite prosecution of this application, the Examiner is invited to contact Eugene T. Perez (Reg. No. 48,501) at the offices of Birch, Stewart, Kolasch & Birch, LLP.

Dated: May 25, 2005

Respectfully submitted,

By _____

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Attachment: Exhibit 1 - *Carbohydrate Research,* "Structure of a fucoidan from the brown seaweed *Fucus evanescens* C.Ag.," Vol. 339, pp. 719-730 (2002)
Exhibit 2 - *Baiasaiensu ta Indasutarii (Biasience and Industry),* "Structures and biological activities of marine algal fucoidans and their oligosaccharides," Vol. 60(6) (2002)).

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Structure of a fucoidan from the brown seaweed *Fucus evanescens* C.Ag.☆

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Received 26 September 2001; received in revised form 20 January 2002; accepted 16 February 2002

Abstract

A fucoidan consisting of L-fucose, sulfate and acetate in a molar proportion of 1:1.23:0.36 was isolated from the Pacific brown seaweed *Fucus evanescens*. The structures of its desulfated and de-*O*-acetylated derivatives were investigated by 1D and 2D ^{1}H and ^{13}C NMR spectroscopy, and the data obtained were confirmed by methylation analysis of the native and desulfated polysaccharides. The fucoidan was shown to contain a linear backbone of alternating 3- and 4-linked α-L-fucopyranose 2-sulfate residues: →3)-α-L-Fuc*p*(2SO_3^-)-(1→4)-α-L-Fuc*p*(2SO_3^-)-(1→. Additional sulfate occupies position 4 in a part of 3-linked fucose residues, whereas a part of the remaining hydroxyl groups is randomly acetylated.

Keywords: Fucoidan; Fucan NMR; Disaccharide repeating unit; Seaweed; Brown algae; *Fucus evanescens*

1. Introduction

Natural polysaccharides built up essentially of sulfated α-L-fucose residues are known as fucoidans.[2] They are present in brown algae and some echinoderms. Fucoidans have been extensively studied due to their diverse biological activities, since they are potent anticoagulant,[3,4] antitumor,[5] and antiviral[6,7] agents. In addition, they can act as ligands for selectins,[8,9] protect the gastric mucosa against the proteolytic activity of gastric juice,[10] block mammalian fertilization,[11] etc. The relationships between structure and biological activities in fucoidans are not clearly established due to many difficulties connected with determination of the fine structure of polysaccharides.

Sulfated fucans isolated from echinoderms have usually linear backbones and regular sulfation patterns resulting in the formation of oligosaccharide repeating units.[12] The structures of these repeating units can be determined unambiguously, especially by using high-field NMR spectroscopy, and hence, correlation between structures and biological action of polysaccharides may be outlined.[13] Unfortunately, the structures of algal fucoidans are much more complicated. The algal polysaccharides are usually heterogeneous and branched, they may contain additional monosaccharide constituents and acetyl groups, the sulfation pattern is not regular, and as a result, chemical methods of structural analysis, as well as NMR spectra of native algal fucoidans, usually give only partial information on their structures. Controversial data may be found in the literature even about the structure of the most carefully studied fucoidan from *Fucus vesiculosus*, which is commercially available.[14–16] It is clear that structures of algal fucoidans vary with the algal species, but their possible structural diversity is also poorly understood. Only recently it was shown that representatives of the orders Chordariales and Laminariales (Phaeosporophyceae) may contain polysaccharides with a linear backbone built up of (1→3)-linked α-L-fucopyranose residues.[17–19] This backbone may have single branches at position 2 of several fucose residues (as α-D-glucopyranosyluronic acid residues in *Cladosiphon okamuranus*[18] or α-L-fucopyranosyl residues in *Chorda filum*[19]) resulting in

☆ Polysaccharides of algae, Part 56. For Part 55, see Ref. 1.

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PII: S0008-6215(02)00053-8

the formation of quasi-regular carbohydrate chains with hexasaccharide repeating units; but in native fucoidans this regularity is masked by random sulfation and acetylation. In contrast, fucoidans from two representatives of the order Fucales (Cyclosporophyceae), namely, *Ascophyllum nodosum* and *F. vesiculosus*, were shown to have a backbone built up of alternating (1→3)- and (1→4)-linked α-L-fucopyranose residues.[16,20] It is very probable that the difference in backbone structures reflects the fundamental difference in fucoidan biosynthesis in the two different classes of brown algae, Phaeosporophyceae and Cyclosporophyceae, respectively, but such a statement requires confirmation by additional examples of fucoidans with definitely elucidated structures.

The present work is devoted to the structural analysis of a fucoidan isolated from the next representative of the order Fucales (Cyclosporophyceae, Phaeophyta), the Pacific brown alga *Fucus evanescens* C.Ag.

2. Results and discussion

Isolation of fucoidan.—Before the extraction of polysaccharides, the algal biomass was pretreated with a MeOH–$CHCl_3$–water mixture to remove pigments and other low-molecular weight compounds.[21] Water-soluble polysaccharides were then extracted from defatted biomass with aqueous calcium chloride at 85 °C, acid polysaccharides were precipitated from the extract by the action of hexadecyltrimethylammonium bromide (Cetavlon) and transformed into water-soluble sodium salts. The resulting crude fucoidan (F) was purified and fractionated by ion-exchange chromatography on DEAE-Sephacel using aqueous sodium chloride of increasing concentration as eluant. The yields and composition of five fucoidan fractions obtained are given in Table 1. Fraction F_4, which was essentially a homofucan sulfate containing fucose and sulfate in a molar ratio of about 1:1.23 and only traces of other monosaccharide constituents, was subjected to structural analysis.

Preliminary characterization and chemical modifications of F_4.—The IR-spectrum of F_4 contained an intense absorption band at 1240 cm^{-1} (S=O) common to all the sulfate esters. An additional sulfate absorption band at 824 cm^{-1} (C–O–S, secondary equatorial sulfate) and a relatively small shoulder at 845 cm^{-1} (C–O–S, secondary axial sulfate) indicated that the majority of sulfate groups occupy positions 2 and/or 3, and only a minor part of sulfate is located at position 4 of fucopyranose residues. An absorption band at 1720 cm^{-1} revealed the possible presence of *O*-acetyl groups in this polysaccharide.

Like many other native algal fucoidans, fraction F_4 had a very complex ^{13}C NMR spectrum, which was difficult to interpret completely (Fig. 1). It contained several intense signals in the anomeric (97–102 ppm) and high-field (16.5–16.7 ppm) regions, which are typical of α-fucopyranosides. The signals at 19–20 ppm confirmed the presence of *O*-acetyl groups. Unfortunately, the ^1H NMR spectrum of F_4 was poorly resolved, so we could not apply 2D procedures to assign other resonances in the ^{13}C NMR spectrum of native polysaccharide.

Several chemical modifications were carried out to simplify the structure of F_4. Three modified polysaccharide preparations were obtained as the result of desulfation (deS), deacetylation (deAc), and both desulfation and deacetylation (deSdeAc). Molar proportions of constituents and specific optical rotation values of F_4 and modified preparations are given in Table 2. Deacetylation was carried out by treatment of polysaccharides with aqueous ammonia.[19] A solvolytic desulfation procedure[22] was used to remove sulfate groups, since acid methanolysis usually results in deep degradation of fucoidans.[17] The yield of desulfated polysaccharide (deS) was 62.3% from theoretical value. The preparation still contained about 7% of residual sulfate, but attempts to split it by additional solvolytic treatment resulted in considerable loss of the material. High negative values of optical rotation of all the four preparations were consistent with α configuration of L-fucopyranose residues in these polysaccharides.

Table 1
Yields and composition of fucoidan fractions obtained by ion-exchange chromatography of crude fucoidan (F)

Fraction	Yield (% of F)	Neutral monosaccharides (%)					SO_3Na (%)	Uronic acids (%)
		Fuc	Xyl	Gal	Man	Glc		
F_1	3.9	35.4	6.1		0.8	4.0	n.d.	n.d.
F_2	2.6	10.7	17.4	3.0	3.7	1.1	19.6	15.6
F_3	21.4	33.2	8.1	4.5	3.5		28.9	11.4
F_4	47.4	58.7	1.6	1.6			46.5	
F_5	4.5	34.0	3.8	5.4			32.5	

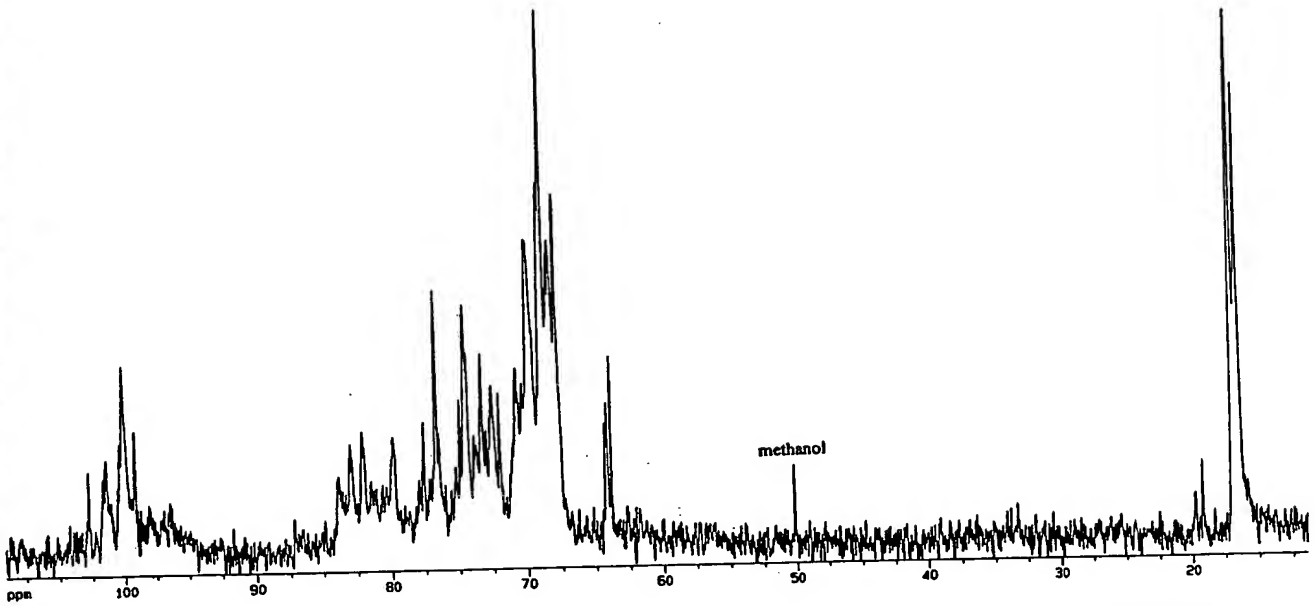


Fig. 1. ^{13}C NMR spectrum of native fucoidan F_4 (recorded at 333 K, the carbonyl region is not shown).

NMR analysis of polysaccharide preparations deSdeAc, deAc, and deS.—Both ^1H (Fig. 2(C)) and ^{13}C (Fig. 3) NMR spectra of desulfated and de-*O*-acetylated polysaccharide (deSdeAc) were resolved enough to apply 2D spectroscopy for the assignment of resonances in the 1D spectra. COSY and TOCSY (Fig. 4) spectra revealed the presence of α-fucose and β-xylose residues in the molecule, the former ones being of two types, **A** and **B**, differing in the mode of substitution. NOESY (Fig. 5) and ROESY spectra showed that all the fucose residues **A** (see Scheme 1 and Tables 3 and 4) were linked to C-4 of residues **B** (correlation peak 5.00/3.90 ppm), whereas all the residues **B** were linked to C-3 of residues **A** (correlation peaks 5.11/3.94 and 5.11/4.03 ppm). Analysis of the HSQC spectrum confirmed substitution of residues **A** at position 3 (downfield location of C-3 resonance at 77.2 ppm) and residues **B** at position 4 (signal C-4 at 81.2 ppm). Finally, type of substitution in these residues was confirmed by HMBC spectrum, where correlation peaks 5.00/81.2 and 5.11/77.2 ppm were observed.

Analysis of β-xylose signals present in 2D spectra revealed (1→4)-linked β-xylopyranose residues only (Table 5, cf.[28]); the subspectrum of terminal xylose residues was not observed. There were no correlation peaks for anomeric protons of the xylose residues with any protons of fucose residues in the NOESY or ROESY spectra. It was concluded that our fucoidan preparation contained a small amount of (1→4)-β-xylan, which was accidentally not separated during the purification steps. Signals corresponding to this xylan were observed only in the spectra of desulfated polysaccharides (deSdeAc and deS), where the relative xylose content was increased due to degradation of some fucoidan molecules under solvolytic desulfation conditions (Table 2). In contrast, spectra of sulfated preparations F_4 and deAc contained practically no signals belonging to xylose residues. Attempts to obtain from the spectra some information about the structural significance of galactose, another minor component of F_4, were unsuccessful.

Thus, according to spectral evidence, desulfated and de-*O*-acetylated fucoidan (deSdeAc) has a linear chain of alternating (1→3)- and (1→4)-linked α-L-fucopyranose residues (Scheme 1, structure **1**). To our knowledge, such a polysaccharide is obtained for the first time. It is interesting to compare its ^{13}C NMR spectrum with the spectra of some related polysaccharides and model compounds (Table 4). As expected, there are marked differences in the positions of anomeric and several other signals in our polysaccharide and linear α-L-fucopyranans of algal or invertebrate origin, containing (1→3) or (1→4) linkages only.[13] The chemical shifts in the spectrum of deSdeAc coincided more satisfactorily with values calculated according to additive

Table 2
Composition (molar proportions) and optical rotation of polysaccharide preparations

Sample	Fuc	Xyl	Gal	SO_3Na	$[\alpha]_D^{24}$ (° in H_2O)
F_4	44	1	1	54	−141.0
deAc	43	1	1	55	−136.0
deS	77	4	4	15	−198.8
deSdeAc	76	6	9	9	−177.0

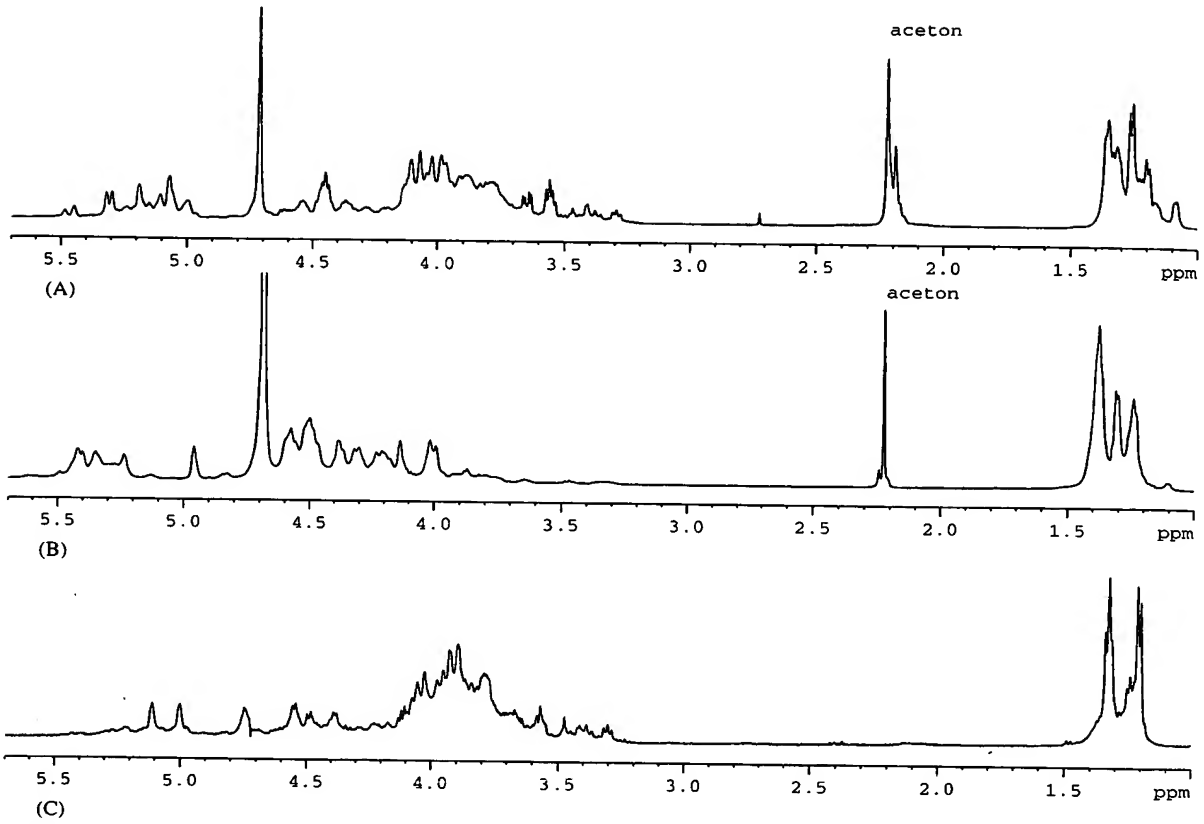


Fig. 2. ^{1}H NMR spectra of desulfated (deS, A), deacetylated (deAc, B), and desulfated and deacetylated polysaccharide (deSdeAc, C).

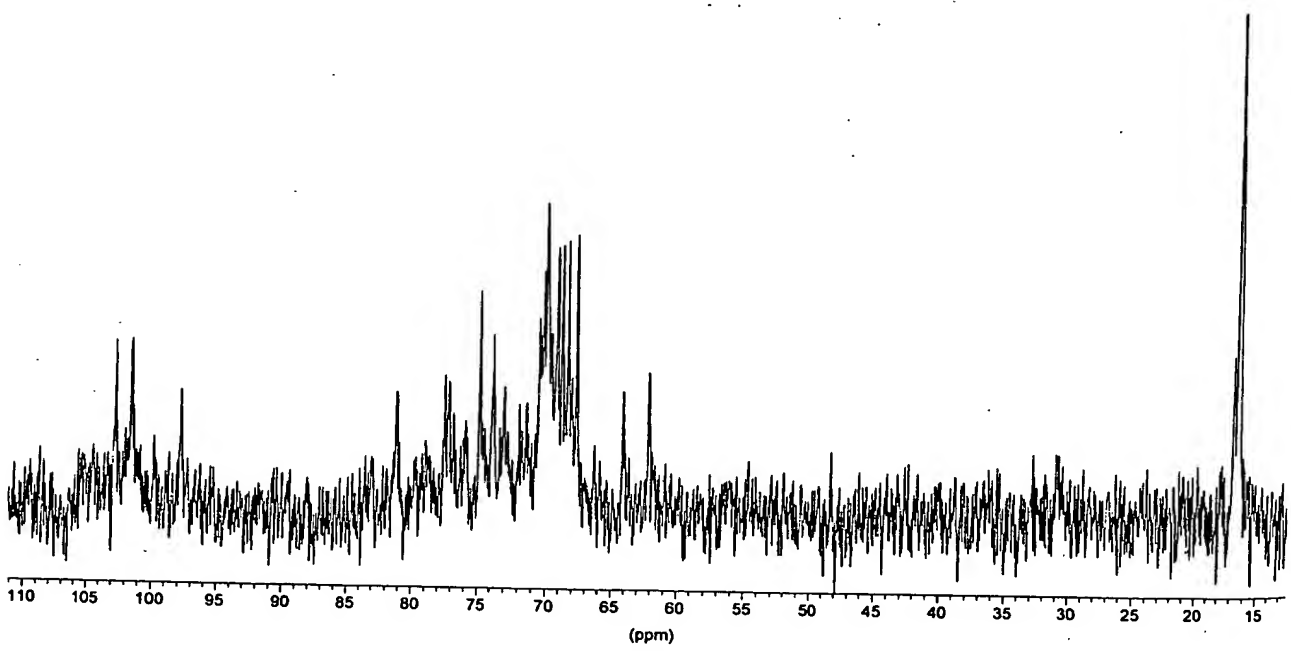


Fig. 3. ^{13}C NMR spectrum of desulfated and deacetylated polysaccharide (deSdeAc).

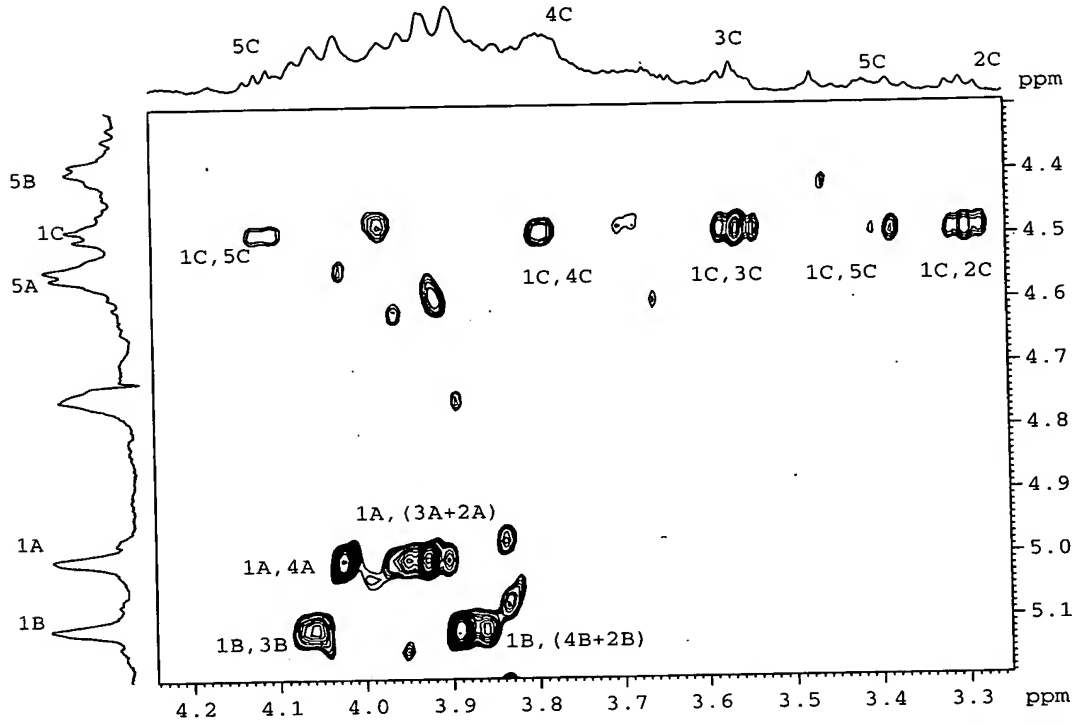


Fig. 4. A part of 2D TOCSY spectrum of desulfated and deacetylated polysaccharide (deSdeAc). Abbreviations: A, 3-linked α-L-fucopyranose; B, 4-linked α-L-fucopyranose; C, 4-linked β-D-xylopyranose.

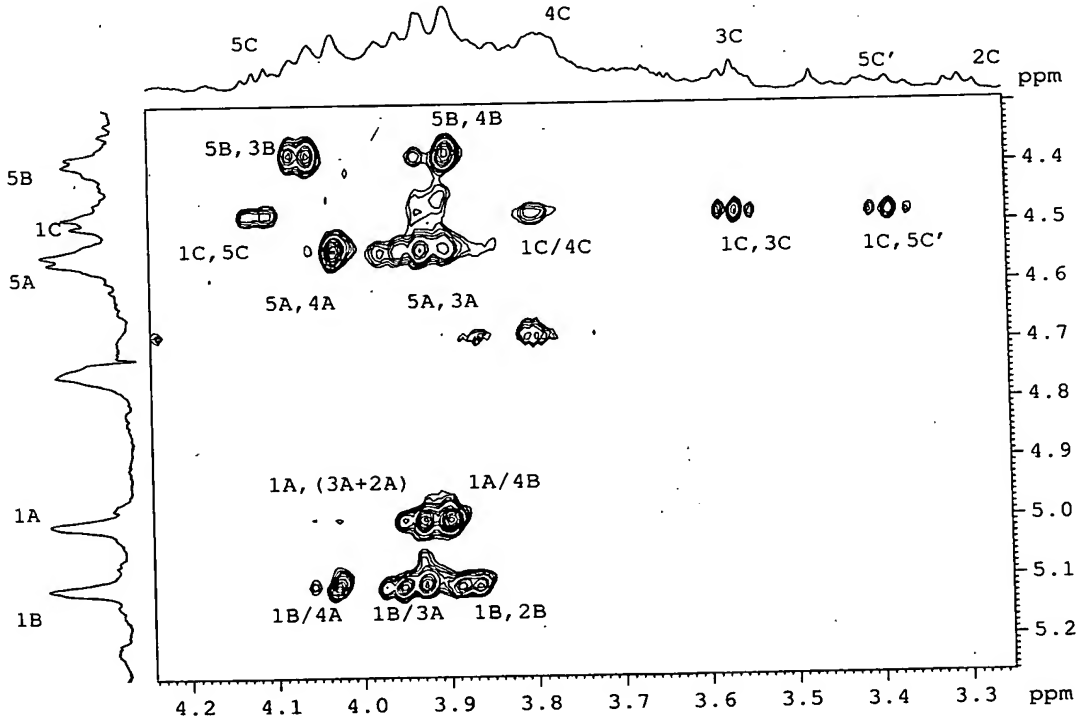
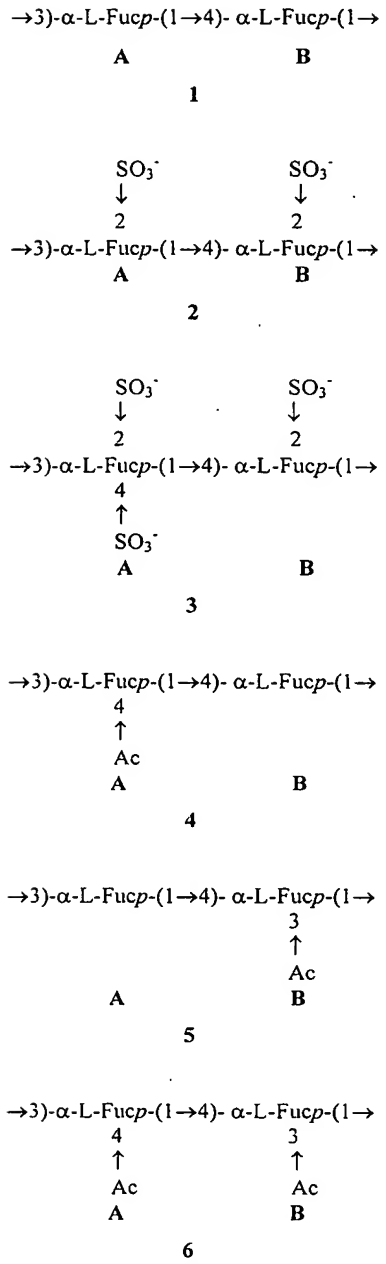


Fig. 5. A part of 2D NOESY spectrum of desulfated and deacetylated polysaccharide (deSdeAc). A, B, C, as in Fig. 4.



Scheme 1.

schemes[19,26] using ^{13}C NMR spectral data for synthetic model (1→3)- and (1→4)-α-L-fucobiosides and respective spectral glycosylation effects[27] (Table 4), but even in this case two remarkable deviations (each of 1.4 ppm) were observed for C-3 (unit **A**) and C-1 (unit **B**), which are involved in the (1→3)-bridge between units **A** and **B**. Most probably, this noticeable deviation of experimental and calculated chemical shift values in the spectrum of deSdeAc is connected with the difference of conformations of model disaccharides and respective fragments within the polysaccharide chain.

All the signals in ^{1}H (Fig. 2(B)) and ^{13}C (Fig. 6) NMR spectra of de-*O*-acetylated polysaccharide (deAc) were assigned using 2D techniques, as above (Table 6). As evidenced from the low-field shifts of H-2 and C-2 resonances in both 3- and 4-linked α-L-fucopyranose residues, all positions 2 in the polysaccharide were sulfated. Low-field shifts of H-4 and C-4 of some 3-linked residues showed that additional sulfate occupies position 4. According to the relative intensities of the corresponding signals in the assigned ^{1}H NMR spectrum, the proportion between structures **2** and **3** (see Scheme 1) was approximately 1:2.

NMR spectra of desulfated fucoidan (deS) (Figs. 2(A) and 7) were analyzed similarly to estimate the molar proportion of fucose and acetate (1:0.36) and to localize the positions of *O*-acetyl groups. It was found that O-4 of 3-linked residues and O-3 of 4-linked residues may be both free or acetylated, as followed from the low-field shifts of corresponding proton and carbon resonances (Table 7), to give structures **4**–**6** (see Scheme 1). The contents of each structure given in Table 7 were calculated from the ^{1}H NMR spectrum of deS.

Methylation analysis.—Methylation of polysaccharides was used to confirm the spectral data on their structure. Native fucoidan (F_4) and desulfated fucoidan (deS) were methylated with methyl iodide in the presence of sodium hydroxide in methyl sulfoxide.[29] F_4 was methylated in the form of both sodium and pyridinium (to enhance solubility) salts, but results of methylation were the same. Methylated polysaccharides were hydrolyzed, and the resulting mixtures of partially methy-

Table 3
^{1}H NMR data for desulfated, deacetylated fucoidan (deSdeAc)

Structure	Residue	^{1}H chemical shifts (ppm)					
		H-1	H-2	H-3	H-4	H-5	H-6
1	→3)-α-L-Fuc^{A}p-(1→	5.00	3.91	3.94	4.03	4.55	1.20
	→4)-α-L-Fuc^{B}p-(1→	5.11	3.89	4.06	3.90	4.39	1.32

Table 4
^{13}C NMR data for desulfated, deacetylated fucoidan (deSdeAc, structure **1**) and some related polysaccharides

Sample	Residue	^{13}C chemical shifts (ppm)					
		C-1	C-2	C-3	C-4	C-5	C-6
Desulfated fucoidan from *C. filum*[19]	→3)-α-L-Fuc*p*-(1→3)-	96.9	67.7	76.3	69.8	67.8	16.5
Desulfated fucoidan [a] from sea cucumber *Ludwigothurea grisea*[23,24]	→3)-α-L-Fuc*p*-(1→3)-	96.8	67.5	76.1	69.6	67.5	16.5
Desulfated fucoidan [a] from sea urchin *Arbacia lixula*[24,25]	→4)-α-L-Fuc*p*-(1→4)-	101.4			80.6		16.5
Experimental and calculated[19,26,27] (in parentheses) chemical shifts and their differences [in brackets] for structure **1**	→3)-α-L-Fuc$\overset{A}{p}$-(1→4)	101.6	68.3	**77.2**	70.2	67.7	16.4
		(101.9)	(67.6)	**(75.8)**	(69.3)	(67.5)	(16.5)
		[−0.3]	[0.7]	**[1.4]**	[0.9]	[0.2]	[−0.1]
	→4)-α-L-Fuc$\overset{B}{p}$-(1→3)	**97.8**	69.2	70.0	81.2	68.8	16.4
		(96.4)	(69.4)	(70.4)	(81.4)	(68.3)	(16.5)
		[1.4]	[−0.2]	[−0.4]	[−0.2]	[0.5]	[−0.1]

[a] Values corrected for the constant difference of −1.5 ppm.

Table 5
NMR data for xylose residues in desulfated polysaccharides (deSdeAc and deS)

Residue	^{1}H chemical shifts (ppm)					
	H-1	H-2	H-3	H-4	H-5	H-5′
→4)-β-D-Xyl*p*-(1→	4.48	3.30	3.56	3.80	4.12	3.38
	^{13}C chemical shifts (ppm)					
	C-1	C-2	C-3	C-4	C-5	
	102.8	73.8	74.8	77.5	64.1	

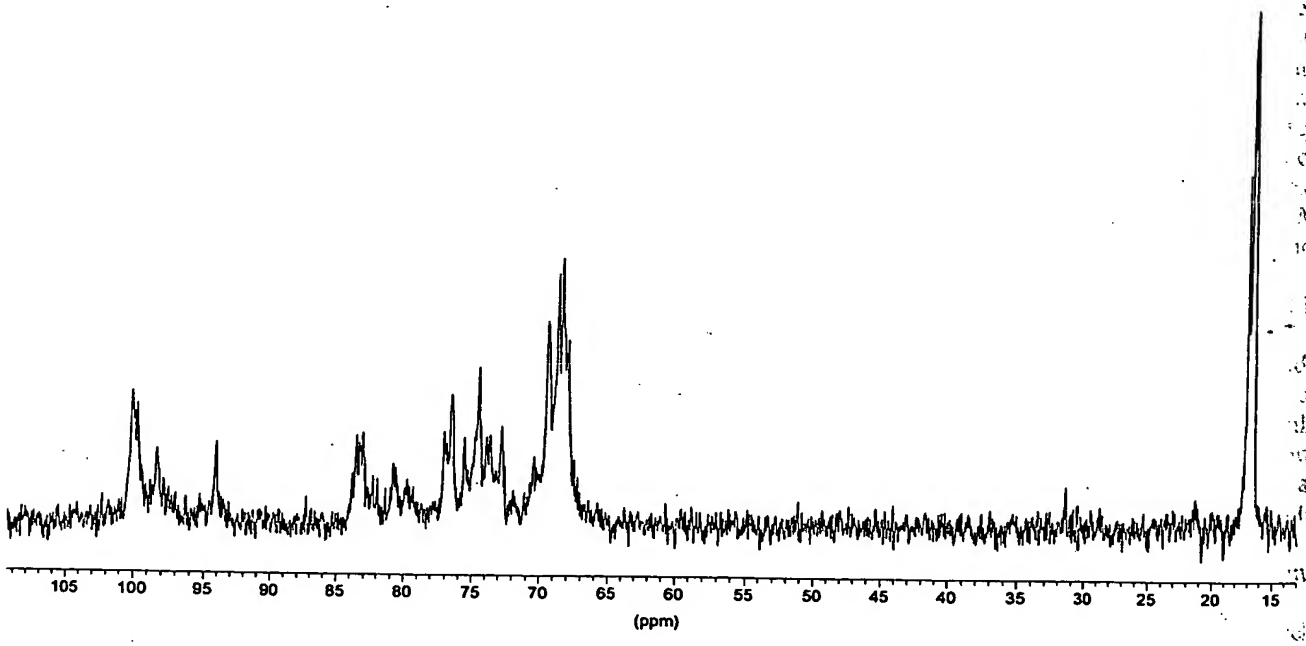


Fig. 6. ^{13}C NMR spectrum of deacetylated polysaccharide (deAc).

Table 6
NMR data for deacetylated fucoidan (deAc)

Structure	Residue	^{1}H chemical shifts (ppm)					
		H-1	H-2	H-3	H-4	H-5	H-6
2 (38.5%)	**A** →3)-α-L-Fuc*p*2SO_3^--(1→	5.23	4.58	4.18	4.13	4.48	1.24
	B →4)-α-L-Fuc*p*2SO_3^--(1→	5.42	4.50	4.22	3.99	4.51	1.41
3 (61.5%)	**A** →3)-α-L-Fuc*p*2,4SO_3^--(1→	5.35	4.57	4.32	4.96	4.54	1.32
	B →4)-α-L-Fuc*p*2SO_3^--(1→	5.40	4.47	4.37	4.02	4.38	1.38
		^{13}C chemical shifts (ppm)					
		C-1	C-2	C-3	C-4	C-5	C-6
2 (38.5%)	**A** →3)-α-L-Fuc*p*2SO_3^--(1→	100.2	74.5	72.8	69.4	68.3	16.7
	B →4)-α-L-Fuc*p*2SO_3^--(1→	94.2	76.5	68.4	83.1	68.7	16.8
3 (61.5%)	**A** →3)-α-L-Fuc*p*2,4SO_3^--(1→	100.2	75.6	73.7	80.8	68.7	17.1
	B →4)-α-L-Fuc*p*2SO_3^--(1→	98.4	76.5	68.4	83.1	69.4	16.8

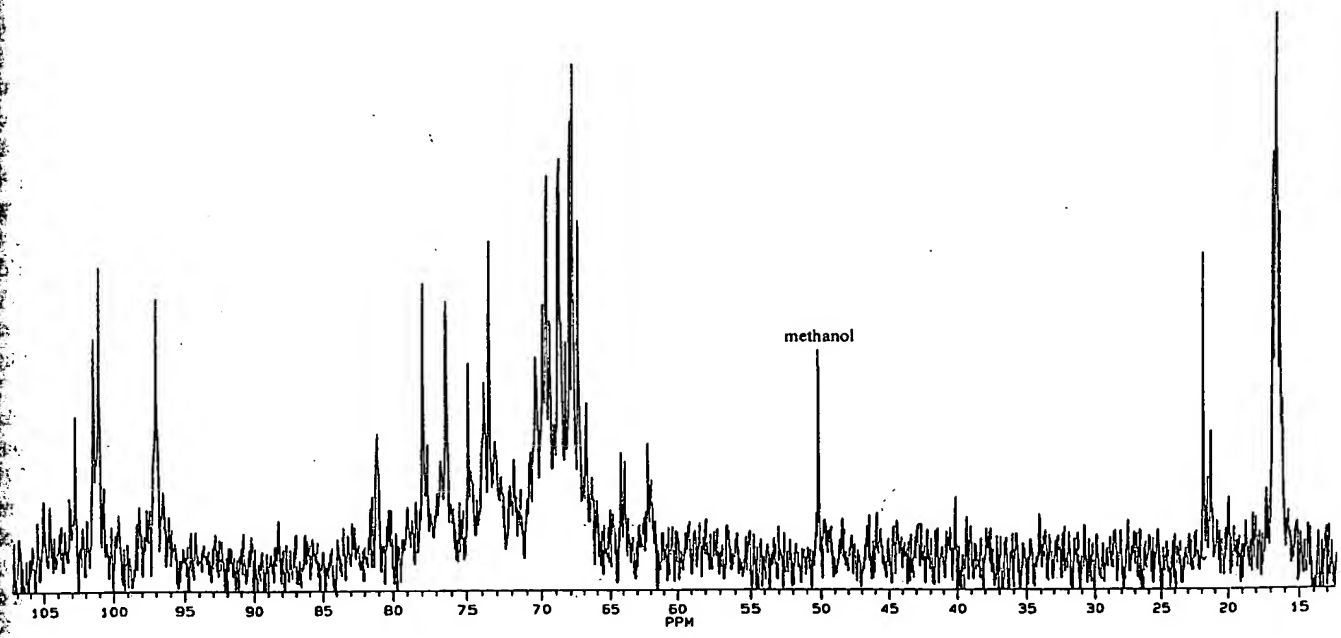


Fig. 7. ^{13}C NMR spectrum of desulfated polysaccharide (deS) (the carbonyl region is not shown).

Table 7
NMR data for desulfated fucoidan (deS)

Structure	Residue	^{1}H chemical shifts (ppm)					
		H-1	H-2	H-3	H-4	H-5	H-6
1 (39%)	**A** →3)-α-L-Fuc*p*-(1→	5.00	3.93	3.95	4.02	4.54	1.20
	B →4)-α-L-Fuc*p*-(1→	5.16	4.07	4.05	3.88	4.37	1.31
4 (16%)	**A** →3)-α-L-Fuc*p*4OAc-(1→	5.05	3.99	4.14	5.45	4.71	1.08
	B →4)-α-L-Fuc*p*-(1→	5.16	4.07	4.05	3.88	4.37	1.31
5 (34%)	**A** →3)-α-L-Fuc*p*-(1→	5.07	3.98	4.01	4.07	4.44	1.26
	B →4)-α-L-Fuc*p*3OAc-(1→	5.18	4.12	5.30	4.11	4.45	1.36
6 (11%)	**A** →3)-α-L-Fuc*p*4OAc-(1→	5.11	4.03	4.21	5.48	4.61	1.16
	B →4)-α-L-Fuc*p*3OAc-(1→	5.18	4.12	5.30	4.11	4.45	1.36
		^{13}C chemical shifts (ppm)					
		C-1	C-2	C-3	C-4	C-5	C-6
1 (39%)	**A** →3)-α-L-Fuc*p*-(1→	101.4	68.2	77.2	70.0	67.4	16.1
	B →4)-α-L-Fuc*p*-(1→	96.5	69.1	70.2	81.1	68.5	16.1
4 (16%)	**A** →3)-α-L-Fuc*p*4OAc-(1→	101.0	68.0	73.8	71.0	66.1	16.0
	B →4)-α-L-Fuc*p*-(1→	96.5	69.1	70.2	81.1	68.5	16.1
5 (34%)	**A** →3)-α-L-Fuc*p*-(1→	101.0	67.9	76.6	71.0	66.0	16.4
	B →4)-α-L-Fuc*p*3OAc-(1→	97.0	66.9	72.4	77.6	68.7	16.2
6 (11%)	**A** →3)-α-L-Fuc*p*4OAc-(1→	101.0	68.7	73.8	71.0	66.0	16.4
	B →4)-α-L-Fuc*p*3OAc-(1→	97.0	66.9	72.4	77.6	68.7	16.2

lated monosaccharides were analyzed as alditol acetates by GLC-MS.[30]

The molar ratios of partially methylated fucitol acetates obtained for desulfated fucoidan (deS) were as follows: acetates of 2,3,4-tri-*O*-methyl-:2,3-di-*O*-methyl-:2,4-di-*O*-methyl-:2-*O*-methyl-fucitol, 7:46:38:9. These results were consistent with the (1→3),(1→4)-backbone of fucoidan. A rather high content of terminal nonreducing fucose residues may be explained by a marked degradation of fucoidan backbone during the

desulfation procedure. Non-equal proportions of 3- and 4-linked fucose, as well as the presence of 2-*O*-methyl-fucose, are possibly due to the presence of residual sulfate at O-4 of some 3-linked fucose residues. Axial sulfate groups seem to be more resistant to solvolysis than equatorial ones: the presence of some 4-sulfated material after solvolytic desulfation of a sulfated fucan from sea cucumber was reported previously.[23]

There were three main components in the products of methylation of native fucoidan (F_4): acetates of 3-*O*-methyl-fucitol, 4-*O*-methyl-fucitol and fucitol in approximately equal amounts. Comparison of these data with the results of methylation of deS confirmed the conclusion that sulfate groups occupy position 2 in all the fucose residues. The presence of non-methylated fucitol acetate was attributed to 3-linked fucose-2,4-disulfate residues in the native polysaccharide.

3. Conclusion

Taking into account the results of spectral and chemical investigation, it may be concluded that the fucoidan isolated from the Pacific brown alga *F. evanescens* has a linear backbone of alternating 3- and 4-linked α-L-fucopyranose 2-sulfate residues. It means that the basic structure of the polysaccharide is regular and contains disaccharide-repeating units. In the native polysaccharide, this regularity is masked by partial sulfation of O-4 in 3-linked residues and by random acetylation of the remaining hydroxyl groups. A polysaccharide having similar backbone was found recently in *A. nodosum*.[16] It has a slightly different overall sulfation pattern, so direct comparison of its NMR spectra with those of our preparations is not possible. It should also be noted that its structural analysis was carried out, using an oligosaccharide fraction isolated from partial hydrolysis products of the starting polysaccharide with the yield of only 2%. Therefore, our data on the presence of alternating sequence of 3- and 4-linked α-L-fucopyranose residues as a backbone of fucoidans are more reliable. Complete assignments of resonances in the NMR spectra of this basic structure and of its sulfated and acetylated derivatives may be used for characterization of other fucoidans, which will be isolated from other species of brown seaweeds. Recently it was shown that some more complex sulfated heteropolysaccharides of brown seaweeds may also contain fucan chains consisting of 3- and 4-linked α-L-fucopyranose residues.[31] Further investigations should indicate whether the presence of alternating sequences found in *A. nodosum* and *F. evanescens* is a characteristic feature of fucoidans from all the algae belonging to the order Fucales.

4. Experimental

General methods.—Quantitative determination of neutral monosaccharides after hydrolysis of polysaccharide samples in 2 M CF_3COOH, 8 h at 100 °C, was performed using GLC of acetylated alditols and myo-inositol as an internal standard.[32] Quantitative determination of uronic acids by color reaction with concd H_2SO_4 and 3,5-dimethylphenol was carried out as described earlier.[33] Sulfate was estimated turbidimetrically[34] after hydrolysis of polysaccharides in 2 M CF_3COOH as above. Fucose was determined with concd H_2SO_4 and L-cysteine hydrochloride.[35]

GLC analyses were carried out with a Hewlett–Packard 5890A chromatograph.[17] IR spectra of polysaccharides were recorded with Perkin–Elmer 577 spectrophotometer in KBr pellets. Optical rotations were measured using a JASCO DIP-360 polarimeter for 0.9% solutions in water.

NMR spectroscopy.—The spectra were recorded using a Bruker DRX-500 spectrometer at 303 K (at 333 K for native fucoidan F_4). Samples were deuterium-exchanged by lyophilization three times with D_2O and then examined as 2–3% solutions in 99.97% D_2O, acetone (δ_H 2.225 ppm) and methanol (δ_C 50.15 ppm) were taken as the internal standards. The data were acquired and performed using XWINNMR 2.1 version. The parameters used for 2D experiments were as follows: COSY [512 × 1024 data matrix; zero-filled to 1024 data points in t_1; four scans per t_1 value; spectral width 2400 Hz; recycle delay 1 s; unshifted sine-square-bell filtering in t_1 and t_2]; ROESY [512 × 1024 data matrix; zero-filled to 1024 data points in t_1; 16 scans per t_1 value; spectral width 2400 Hz; mixing time 200 ms; shifted sine-squared filtering in t_1 and t_2]; NOESY [512 × 1024 data matrix; zero-filled to 1024 data points in t_1; eight scans per t_1 value; spectral width 2400 Hz; mixing time 600 ms; shifted sine-squared filtering in t_1 and t_2]; TOCSY [512 × 1024 data matrix; zero-filled to 1024 data points in t_1; eight scans per t_1 value; the duration of the MLEV17 spin-lock was 60 ms]; HSQC [256 × 1024 data matrix; zero-filled to 512 data points in t_1; 40 scans per t_1 value; spectral width in t_1 2400 Hz and in t_2 11970 Hz; recycle delay 1.0 s; shifted sine-squared filtering in t_1 and t_2]; HMBC [512 × 1024 data matrix; 56 scans per t_1 value; spectral width in t_2 2400 Hz and t_2 22680 Hz; recycle delay 1.0 s; optimization of the experiment for coupling constant 8 Hz].

Isolation of fucoidan.—The alga *F. evanescens* was collected from the littoral of Iturup island (Kuril Islands) in August of 1997, soaked in acetone and dried in air. The milled algal biomass was treated at rt with a 4:2:1 MeOH–$CHCl_3$–water mixture to remove colored matter, filtered and vacuum dried. Then the mixture of defatted algal biomass (15 g) and 2% aq $CaCl_2$ (4 × 150 mL) was mechanically stirred at 85 °C for 5 h. An aq

hexadecyltrimethylammonium bromide solution (10%, 50 mL) was added to the combined extracts. The precipitate formed was centrifuged, washed with water, stirred with 20% ethanolic NaI solution (3 × 60 mL) for 2–3 days at rt, washed with EtOH, and dissolved in water. The solution was dialyzed and lyophilized to give crude fucoidan fraction (F) as sodium salt, yield 1.94 g (12.9% of dry defatted biomass); composition: fucose, 42.4%; SO_3Na, 35.8%; xylose, 3.6%; galactose, 2.9%; mannose, 1.2%; glucose 0.7%. An aqueous solution of F (1.54 g in 50 mL) was placed on a column (24 × 4 cm), containing DEAE-Sephacel (Pharmacia) in Cl^--form, and eluted with water followed by NaCl solutions of increasing concentration (0.5, 1.0, 1.5 and 2.0 M), each time up to the absence of a positive reaction of eluate for carbohydrates[36] with phenol and concd H_2SO_4. All the solutions obtained were dialyzed and lyophilized, yields of fractions F_1–F_5 being 0.06, 0.04, 0.33, 0.73, and 0.07 g, respectively. Composition of these fractions is given in Table 1.

Desulfation of fucoidan.—To convert F_4 into pyridinium salt an aqueous solution of fucoidan was passed through a Dowex 50W × 4 (PyH^+-form) column, the eluate was concentrated and freeze-dried. Solvolytic desulfation of F_4 (as pyridinium salt) was carried out as described earlier.[17] Yield of desulfated fucoidan (deS) was 80 mg from 240 mg of the starting material, residual SO_3Na (6.7%).

De-O-acetylation of polysaccharides.—Samples of F_4 and deS were treated with aqueous ammonia at 37 °C to remove acetyl groups.[19]

Methylation analysis of polysaccharides.—Methylation of fucoidans followed by hydrolysis and GLC-MS of partially methylated fucitol acetates was performed as previously described.[17,19]

Acknowledgements

This work was supported by the Scientific Council 'Chemistry and Technology of Renewable Plant Raw Materials' of Russian Academy of Sciences, grant 8.1.14, and by Russian Foundation of Basic Research, grant 01-03-33059. The authors are grateful to Drs T.N. Zvyagintseva and V.V. Sova (Pacific Institute of Bioorganic Chemistry, Far-Eastern Branch of the Russian Academy of Sciences, Vladivostok, Russia) for the gift of a sample of *Fucus evanescens*.

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シリーズ ―見えてきたグライコテクノロジーの実用への道⑤

海藻由来フコイダンとそのオリゴ糖の構造と生物活性

Structures and biological activities of marine algal fucoidans and their oligosaccharides

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フコイダンには多くの分子種がある。筆者らは種々のフコイダン分解酵素を用いて各種フコイダンオリゴ糖を調製し、それらの構造を決定し、さらにフコイダンの全体構造も決定した。そして、多糖とオリゴ糖の生物活性を比較した。

キーワード：フコイダン、フコイダン分解酵素、フコイダンオリゴ糖、海洋細菌

はじめに

今から100年ほど前に、ドイツの研究者により、*Fucus vesiculosus* というヒバマタの類縁海藻に硫酸化多糖が含まれていることが見いだされフコイジンと命名されたが、その後、多糖の命名の規約により、その硫酸化多糖はフコイダンと呼ばれるようになった。その後、褐藻綱に属する海藻から次々と硫酸化L-フコースを含む多糖が見いだされた。しかし、これらの多糖の分類基準は確立されておらず、総称的にフコイダンと呼ばれることが多かった。実際、フコイダンには多くの分子種があり、それぞれの分子により、L-フコースの結合様式、構成糖、硫酸基の含量等が異なる。しかも一般に、1種の海藻に数種のフコイダンが含まれているので、分子種ごとに分離するのは困難であった。一方、フコイダンには、抗がん作用や抗凝血作用をはじめとする有用な生物活性があるので、それらの活性を担う構造の解明が試みられたが、部分構造や平均構造が提唱された程度で、構造と活性の関係を解明するにはほど遠い状況であった。

筆者らは最初に、ガゴメ（*Kjellmaniella crassifolia*）由来フコイダンのがん細胞に対するアポトーシス誘発作用に着目した。そこで、フコイダン資化性細菌が生産する種々のフコイダン分解酵素を用いてオリゴ糖を調製し、それらのアポトーシス誘発作用を調べ、その作用を担う最小構造単位の解明を試みた。さらに、筆者らが確認したフコイダンのそのほかの生物活性についても、同様の検討を行った。また、この過程で多くのフコイダンオリゴ糖の構造を決定し、数種のフコイダンの全体構造も決定することができた。

1．フコイダンの調製

筆者らは、図1に記載した方法により、種々の褐藻綱に属する海藻からフコイダンを調製した。なお、オキナワモズクおよびモズクは、湿藻体を使用した。本方法を用いて調製したフコイダンは、分子量10万以下のフコイダン、すなわち著しい構造破壊を受けた分子が排除されているため、構造解析および構造が均一なオリゴ糖の調製に適していると考えている。また、各海藻から得られたフコイダンの量を表1に示すが、

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乾燥海藻粉末
　├ ８０％エタノール洗浄、ろ過
エタノール洗浄粉末
　├ 熱水抽出、ろ過
熱水抽出物
　├ アルギン酸リアーゼ処理
　├ 活性炭処理、遠心分離
上清
　├ 限外ろ過 (cut-off 100K)
保持液（高分子画分）
　├ 酸処理（５℃）、遠心分離
上清
　├ 中和、限外ろ過 (cut-off 100K)
保持液（高分子画分）
　├ 凍結乾燥
フコイダン

図１　フコイダンの製造方法

フコイダン含量は、海藻の種だけではなく同種の海藻であっても、その部位、収穫期、個体の成長度等によりかなり変動する。

２．フコイダン資化性細菌の単離

フコイダンの構造決定に利用できる酵素、すなわちフコイダンをオリゴ糖単位で切断できる酵素に関する報告はなかった。そこで、筆者らはフコイダン分解酵素を得るため、海洋微生物をスクリーニングして、数種のフコイダン資化性細菌を得た。これらの細菌の性質および16ＳrDNA配列を調べ、これまでに命名された細菌の諸データと比較したところ、同じ属と認められるものすら見いだせなかったので、表２に示すように命名した。*F. marina* および *F. lyticus* は、異なったフコイダン特異性を示したが、*F. fucoidanolyticus* は、調べた限り、すべての海藻由来フコイダンをある程度資化した。すなわち、本細菌は今回報告する以外にも、多種類のフコイダン分解酵素の生産源として利用できる可能性がある。

表１　各種海藻のフコイダン含有量

海藻	フコイダン含有量 (g/kg 乾燥海藻)
コンブ目海藻	
ガゴメ	40
マコンブ	15
ワカメ(葉状部)	15
ワカメ(胞子葉部)	80
アラメ	70
Ecklonia maxima	40
Lessonia nigrescens	46
ヒバマタ目海藻	
Fucus vesiculosus	70
Ascophyllum nodosum	110
ナガマツモ目海藻	
オキナワモズク	250
モズク	250

３．フコイダン分解酵素の調製およびそれらの酵素を用いて得られたオリゴ糖の構造

単離したフコイダン資化性細菌を、種々のフコイダン含有培地で大量培養して、種々のフコイダン分解酵素を調製した(表２)[1)~4)]。これらの酵素のうち、６種に関しては、すでに大腸菌を用いた生産系を確立した(特許出願済み)。

次に、これらの酵素をそれぞれの基質となるフコイダンに作用させて、それぞれのオリゴ糖を得た。得られたオリゴ糖を精製・単離後、NMR分析等により化学構造を決定したところ、各オリゴ糖は共通の骨格構造を持っており、どのフコイダンにも繰返し構造が存在することを示すものであった。いくつかのフコイダンに関しては、オリゴ糖間の結合様式も解明し、フコイダンの全体構造を決定することができた(図２)。

これまでフコイダンの構造は非常に複雑だといわれ、その化学構造は解明されていなかった。おそらく、分子種の分離が不十分であったり、フコイダン分子内のフコシル結合およびエステル硫酸結合が物理化学的に非常に弱いことなどから、二次的に多種類のフコイダンが生成することが、構造解明を妨げていたと考えられる。前述のように、筆者らがフコイダンの構造を解明できたのは、特定の分子種にしか作用しないエンド型のフコイダン分解酵素を利用することで、①標的となる分子のみを低分子化して混在するほかのフコイダン分子から容易に分離できる、②標的となる分子をその繰返し構造単位に切断するので多糖の構造の推定が容易となる、③酵素反応を中性付近、常温で行うためフコイダンの構造を破壊することがない、④酵素反応により得られる産物は低分子であるためNMR分析が容易となる、などの多くの利点があったためである。

フコイダンも整然とした繰返し構造を持つということを明らかにできた(図２)。

４．種々のフコイダン

筆者らは、これまで６種のフコイダンの存在を明ら

表2　単離したフコイダン資化性海洋細菌、それらが生産するフコイダン分解酵素、およびそれらの基質

細菌の名称	生産が確認されたフコイダン分解酵素	基質	
		由来となる海藻	フコイダン分子種
Fucobacter marina			
	硫酸化フコグルクロノマンナン分解酵素 I *	ガゴメ	硫酸化フコグルクロノマンナン
	硫酸化フコグルクロノマンナン分解酵素 II *	ガゴメ	硫酸化フコグルクロノマンナン
	硫酸化フコガラクタン分解酵素 I *	ガゴメ	硫酸化フコガラクタン
	硫酸化フコガラクタン分解酵素 II *	ガゴメ	硫酸化フコガラクタン
Fucanobacter lyticus			
	硫酸化フカン分解酵素 I *	ガゴメ	硫酸化フカン
	硫酸化フカン分解酵素 II *	ガゴメ	硫酸化フカン
Fucophilus fucoidanolyticus			
	硫酸化グルクロノフカンデアセチラーゼ	オキナワモズク	硫酸化グルクロノフカン
	α-D-グルクロニダーゼ	オキナワモズク	硫酸化グルクロノフカン
	エンド-α-L-フコシダーゼ	オキナワモズク	硫酸化グルクロノフカン
	硫酸化フコグルクロノマンナン分解酵素	*F. vesiculosus*	硫酸化フコグルクロノマンナン
	硫酸化フカン分解酵素	*F. vesiculosus*	硫酸化フカン

＊大腸菌を用いた生産系確立済み

硫酸化フコグルクロノマンナン（ガゴメ由来）
主要オリゴ糖　ΔGA1-2(F(3S)α1-3)Manα1-4(GAβ1-2(F(3S)α1-3)Man)$_n$　n = 0, 1
多糖の主要構造　(-4GAβ1-2(F(3S)α1-3)Manα1-)$_m$

硫酸化フコグルクロノマンナン（*Fucus vesiculosus* 由来）
主要オリゴ糖　ΔGA1-2(Ff(5S)α1-4F(2,3diS)α1-3)Man(6S)
多糖の中での存在形態　-4GA1-2(Ff(5S)α1-4F(2,3diS)α1-3)Man(6S)α1-

硫酸化フコガラクタン（ガゴメ由来）
主要オリゴ糖　Gal(3S)β1-6Gal(3S)β1-6(F(3S)α1-4F(3S)α1-3Galβ1-4)Gal(3S)
多糖の中での存在形態　-6Gal(3S)β1-6Gal(3S)β1-6(F(3S)α1-4F(3S)α1-3Galβ1-4)Gal(3S)β1-

硫酸化フカン（ガゴメ由来）
主要オリゴ糖
F(2,4diS)α1-3F(2,4diS)α1-3(F(3S)α1-2)F(4S)α1-3F(2,4diS)α1-3F(2,4diS)α1-3F(2,4diS)

硫酸化フカン（*Fucus vesiculosus* 由来）
主要オリゴ糖　Fα1-3(F(2S)α1-4F(2,3diS)α1-3)$_n$F(2S)　n = 1, 2, 3, 4

硫酸化グルクロノフカン（オキナワモズク由来）
主要オリゴ糖
(Fα1-3F(4S)α1-3F(4S)α1-3(GAα1-2)F(4-*O*-acetyl)α1-3)$_n$ Fα1-3F(4S)α1-3F(4S)α1-3F　n = 0, 1, 2
多糖の主要構造
(-3Fα1-3F(4S)α1-3F(4S)α1-3(GAα1-2)F(4-*O*-acetyl)α1-)$_m$

図2　酵素消化により得られたフコイダンオリゴ糖の構造およびフコイダンの全体構造
図中略号　F, L-fucose；Ff, L-fucofuranose；GA, D-glucuronic acid；ΔGA, 4, 5-unsaturated D-glucuronic acid；Gal, D-galactose；Man, D-mannose；S, *O*-sulfate

かにした(図2)。ガゴメと *F. vesiculosus* にはともに硫酸化フコグルクロノマンナン(SFGM)が含まれている。両者の主鎖構造は同じだが側鎖構造が異なるため、*F. marina* の酵素では *F. vesiculosus* の SFGM をほとんど分解できない。また、*F. marina* は2種の SFGM 分解酵素を持つが[5]、両者の SFGM-6糖に対する反応速度差が大きいので、両酵素を用いて3糖および6糖を意図的に調製できる[6]。ガゴメには、全フコイダンの数%程度しか硫酸化フコガラクタン(SFG)が含まれていないが、酵素の利用により、このようなマイナー成分の構造も決めることができた。また、ワカメメカブに含まれるフコイダンを SFG 分解酵素により分解して、そのオリゴ糖を調製することもできた。

硫酸化フカン(SF)はガゴメおよび *F. vesiculosus* の主要フコイダンであるが、両者は主鎖構造が異なり、どちらの SF 分解酵素も他方の SF を分解できない。また、ガゴメの SF は特に硫酸化度が高く、分子内フコース残基の水酸基の約90%が硫酸基で置換されている。なお、*F. vesiculosus* 由来 SF の全体構造はま

だ決定できていないが、図2に示す構造は、酸部分加水分解で得られたオリゴ糖の構造と矛盾がない[7]。しかしながら、それ以前に報告されていた平均的構造とはあまり共通点が認められない。これはすなわち、硫酸化度の高いフコイダンの平均的構造を化学的に分析しても、分子の実際の化学構造をとらえるのは困難であることを示している。

硫酸化グルクロノフカン(SGUF)は、オキナワモズクの主要フコイダンであるが、ガゴメSFと比較すると、硫酸含量は約1/5である。そのため、両者の生物活性を比べると大きな違いが見られる。

前述した6種のフコイダンは、ここに述べた海藻だけに含まれるものではない。例えば、*F. marina* 由来SFGM分解酵素およびSFG分解酵素は、多くのコンブ目海藻由来フコイダンに作用してオリゴ糖を生成させる。すなわち、分類学的に近い海藻のフコイダンは構造上の共通点があるといえる。しかもそれらのオリゴ糖は主鎖構造が同じで、結合している硫酸基の位置や数のみが異なるというように、構造と活性の関係を調べる上で非常に有用なオリゴ糖となる可能性が高い。

5: フコイダンとフコイダンオリゴ糖の生物活性

筆者らは、ガゴメ由来フコイダンに、担がん動物の延命、がん細胞のアポトーシス誘発、担がん動物の脾臓細胞のIFN-γおよびIL-12産生増強、各種細胞のHGF産生増強等の有用な生物活性があることを確認し、これらの活性を人々の健康に役立てるため、ガゴメ由来フコイダンを含有する種々の食品(飲料、顆粒、食品素材粉末)を製品化した[8),9)]。フコイダンを含む化粧品も製品化しており、最近の研究で、ガゴメのフコイダンを実験動物の皮膚に塗布すると、紫外線が原因となる老化現象(しわ形成、弾力性低下、コラーゲン生産量低下)を抑制できることも証明できた。また、これらの活性と構造の関係を解明するために、種々のフコイダンおよびそれらのオリゴ糖の生物活性も調べたが、オリゴ糖に断片化すると、ほとんどの生物活性はもとのフコイダンより弱くなった。硫酸化フカンオリゴ糖ではHGF産生増強作用が比較的保持されていたが[10)]、もとのフコイダンをしのぐものではなかった。

なお、これまでに生物活性を調べたオリゴ糖は、構造決定が可能な比較的分子量の小さなものに限られていた。今後、より大きな分子量のオリゴ糖を調べれば、フコイダンと対等あるいはそれ以上の生物活性を持つものを見いだせる可能性もある。

おわりに

筆者らは、現在も新規フコイダン分解酵素の研究開発を進めており、今後さらに多種類のオリゴ糖を得ることが可能になる。図2に示すように、フコイダンオリゴ糖には、構成糖、分岐パターン、硫酸化度、分子サイズなどにおいて広い多様性がある。そのため、ほかの多糖由来オリゴ糖では考えられないような生物活性が見いだせる可能性もある。それぞれのフコイダン由来オリゴ糖も量産できる体制が整いつつある今、オリゴ糖としての有効利用法の開発が強く望まれる。

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